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ENSENAT WASSER, Roberto [ES/ES]; C/ Diosa Tanit 3,  
12A, E-03016 Alicante (ES).

(21) International Application Number: PCT/EP02/00475

(74) Agent: ACKERMANN, Joachim; Postfach 11 13 26,  
60048 Frankfurt (DE).

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(71) Applicant (*for all designated States except US*): CAR-  
DION AG [DE/DE]; Max-Planck-Strasse 15a, 40699  
Erkrath (DE).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): SORIA ESCOMS,  
Bernat [ES/ES]; Urbanización Los Torreones, 10, C/Mu-  
jol 11, E-03540 Alicante (ES). BERNÁ AMOROS, Gen-  
oveva [ES/ES]; C/ Hacienda del Administrador 4, 10C,  
E-03560 Campello, Alicante (ES). REIG MACIÀ, Juan  
Antonio [ES/ES]; Segura 12, 3 Izq, E-03004 Alicante (ES).  
MARTÍN BERMUDO, Franz [ES/ES]; C/ Hacienda del  
Administrador 4, 10C, E-03560 Campello, Alicante (ES).

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(54) Title: PLURIPOTENT ADULT STEM CELLS DERIVED FROM REGENERATIVE TISSUE

(57) Abstract: The present invention provides a pluripotent adult stem cell population derived from regenerative tissue, having alkaline phosphatase activity, high levels of telomerase activity and the ability to form derivatives of all three embryonic germ layers and/or the ability to form embryoid bodies.

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Pluripotent adult stem cells derived from regenerative tissue

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Description

The present invention relates generally to isolated, enriched, mammalian, preferably human, adult stem cell and progenitor cell populations derived from regenerative tissue, having pluripotent stem cell-like characteristics, their differentiation into any cell type, and therapeutic use thereof.

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Regenerative stem cell populations constitute only a small percentage of the total number of cells, but are of immense interest because of their ability to repopulate the body. There is significant commercial interest in these methods because stem cells have a number of clinical uses. Using stem cells for generation of organs and tissues for transplantation provides a promising alternative therapy for diabetes, neuronal disease, liver disease, heart disease and autoimmune disorders, to name a few. Diabetes, for instance is an excellent candidate for stem cell treatment (Soria et al. (2001) Diabetologia 44: 407-415; Soria et al. (2000) Eur. J. Physiol. 440: 1-18; and Berná et al. (2001) Biomed. Pharmacother. 55: 206-212).

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Regenerative stem cells can be found in many adult tissues, participating in normal replacement due to physiological turnover or in response to injury. A mouse model of intestinal stem cells has been developed (Roth et al. (1991) Cell Biol. 88: 9407-9411; and Slorach et al. (1999) J. Cell Sci. 112: 3029-3038).

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Recently, several reports have appeared indicating that the functional plasticity of adult stem cells can be greater than expected. In this regard, neural stem cells from the adult mouse brain produced hematopoietic progeny and incorporated into an embryo, contributing to all the tissues of the resulting chimeric mouse (Bjornson et al. (1999)

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Science 283: 534; and Clarke et al. (2000) Science 288: 1666). Moreover, a recent study showed that clonally derived neural stem cells from mice and humans can produce skeletal myotubes in vitro and in vivo (Galli et al. (2000) Nat. Neurosci. 3: 986). In addition, bone marrow cells can differentiate into myogenic progenitors, myocytes, vascular structures, neurons, or liver cells (Ferrari et al. (1998) Science 279: 1528; Orlic et al. (2001) Proc. Natl. Acad. Sci. USA 98: 10344; Woodbury et al. (2000) Neurosci. Res. 61: 364; and Alison et al. (2000) Nature 404: 257). In addition, stem cells isolated from skeletal muscle have shown hematopoietic potential (Jackson et al. (1999) Proc. Natl. Acad. Sci. USA 96: 14482). These studies argue against the notion that the ability of a somatic stem cell to produce mature cell populations is limited to the range of cell types characteristic of the individual source tissue.

#### Brief summary of the invention

An object of the present invention is to provide isolated pluripotent adult stem cell and progenitor cell populations, derived from regenerative tissue, which can differentiate into any cell type, and methods for isolating and enriching pluripotent adult stem cell and progenitor cell populations.

The object is achieved by means of providing an isolated pluripotent adult stem cell or stem cell population and a composition comprising said pluripotent adult stem cell or stem cell population, derived from regenerative tissue, and having alkaline phosphatase activity, high levels of telomerase activity and the ability to form derivatives of all three embryonic germ layers and/or the ability to form an embryoid body.

The object is further achieved by a method for isolating and enriching the pluripotent adult stem cell population, comprising the steps of: a) isolating a population derived from regenerative tissue containing somatic adult stem cells; b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer; c) selecting and replating colonies which have a pluripotent morphology; wherein the cells of the selected colonies are preferably expanded over 2-100 passages, very particularly over 2-15 passages.

Another object of the present invention is to provide a differentiated cell population and methods for selecting and differentiating the pluripotent adult stem cell population.

The object is achieved by a method for selection and differentiation of a pluripotent adult stem cell population to a desired differentiated cell population, comprising the steps of:

5 a) isolating a population derived from regenerative tissue containing somatic adult stem cells; b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer; c) selecting and replating colonies which have a pluripotent morphology; d) causing differentiation by deleting the fibroblast feeder layer; and e) selecting the cells

10 of said desired cell lineage.

The object is further achieved by a method for selection and differentiation of a pluripotent adult stem cell population to a desired differentiated cell population, comprising the steps of: a) isolating a population derived from regenerative tissue

15 containing somatic adult stem cells; b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer; c) selecting and replating colonies which have a pluripotent morphology; d) transfecting cells in selected colonies to introduce a marker gene that enables the selection of one desired cell lineage from the other cell lineages that result from differentiation of the stem cells; e) causing differentiation by deleting the

20 fibroblast feeder layer and culturing at high density in suspension; and f) selecting the cells of said desired cell lineage based on the marker gene.

The object is further achieved by a method for selection and differentiation of a pluripotent adult stem cell population to a desired differentiated cell population,

25 comprising the steps of: a) isolating a population derived from regenerative tissue containing somatic adult stem cells; b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer; c) selecting and replating colonies which have a pluripotent morphology; d) transfecting cells in selected colonies to introduce a lineage-specific gene for the purpose of driving differentiation toward a chosen cell lineage, and

30 a marker gene for the purpose of selection; e) causing differentiation by deleting the

fibroblast feeder layer and culturing at high density in suspension; and f) selecting the enriched chosen cell lineage based on the marker gene.

Other objects of the invention are to provide a method of treating a disease, comprising administering said pluripotent adult stem cell population or said differentiated cell population to a patient in need thereof, and to provide a therapeutic and/or diagnostic composition comprising said pluripotent or said differentiated cell population.

Cells and cell populations obtained by these steps, not only have pluripotent morphology (see Figure 1B) but, surprisingly, the pluripotent stem cell-like characteristics of alkaline phosphatase activity, high levels of telomerase activity, stage-specific embryonic antigen-3 (SSEA-3) and stage-specific embryonic antigen-4 (SSEA-4) expression (only in the case of human origin), and the ability to form derivatives of all three embryonic germ layers. After the final step, embryoid body (EB) formation can be obtained (see Figure 1D). EBs are described, for instance, by Maltsev et al. (1993) Mech. Dev. 44:41-50.

#### Detailed description of the invention

The present invention relates to the isolation of pluripotent adult stem cell and progenitor cell populations, derived from regenerative tissue, which can differentiate into any cell type, and methods for the isolation and enrichment of pluripotent adult stem cell and progenitor cell populations.

The isolated pluripotent adult stem cell or stem cell population, is derived from regenerative tissue, and has alkaline phosphatase activity, high levels of telomerase activity and the ability to form derivatives of all three embryonic germ layers. The isolated pluripotent adult stem cell population also has the ability to form an embryoid body.

The method for isolating and enriching the pluripotent adult stem cell population, comprises the steps of: a) isolating a population derived from regenerative tissue

containing somatic adult stem cells, preferably human; b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer, preferably human; c) selecting and replating colonies which have a pluripotent morphology; wherein the cells of the selected colonies are preferably expanded over 2-100 passages, more preferably over 5 2-15 passages and most preferably over 10-15 passages. Specific methods for pursuing each step are provided for instance, in the Examples herein.

Preferably, the undifferentiated cells, as obtained in step c), are mammalian cells, and even more preferably, human cells, which have the potential to differentiate into any cell 10 lineage when subjected to differentiating conditions. Furthermore, the undifferentiated cells preferably can proliferate and propagate in vitro, and can maintain an undifferentiated state when cultured up to 15 passages on a fibroblast feeder layer. The undifferentiated cells preferably have a total expansion capacity of more than  $1 \times 10^{10}$ , more preferably of more than  $1 \times 10^{11}$ . The undifferentiated cells express SSEA-3, and 15 preferably can express any other characteristic stem cell markers known in the art.

The regenerative tissue can be selected from the group consisting of peripheral blood, cord blood, bone marrow, brain, skin, retina, hair papilla, muscle, liver, pancreas, epithelium. In a preferred embodiment, the regenerative tissue are epithelia, preferably 20 mammalian intestinal epithelia, most preferably human intestinal epithelia derived from the small intestine, in particular, derived from crypts.

The experiments show that, in particular, adult human intestinal stem cells (HISC) can be induced in vitro to overcome their epithelial commitment and differentiate in other cell 25 types.

Epithelia are usually composed of several distinct cell types, and the ability to form all of them is an aspect of stem cell behavior. The mammalian intestinal epithelium is a rapidly proliferating tissue, with stem cells residing at the crypt base and giving rise to all 30 cell types found within the crypt (Bach et al. (2000) Carcinogenesis 21: 469). Experimental evidence for the regenerative capacity of the adult intestinal epithelium



has been recently demonstrated by growing subcutaneously in immunocompromised mice cultured small intestinal crypts that gradually showed an epithelial differentiation typical of the normal intestine (Booth et al. (1999) *Exp. Cell Res.* 249: 359).

- 5 By "stem cell" is meant, in accordance with the present invention, a cell that can undergo self-renewing cell division, for an indefinite time, to give rise to phenotypically and genotypically identical daughter cells, and can ultimately differentiate into at least one final cell type.
- 10 The quintessential stem cell is the embryonic stem (ES) cell, as it has unlimited self-renewal and multipotent and/or pluripotent differentiation potential, thus possessing the capability of developing into any organ, tissue, or cell type. These cells can be derived from the inner cell mass of the blastocyst, or can be derived from the primordial germ cells from a post-implantation embryo, in which case they are called embryonic germ
- 15 (EG) cells. ES and EG cells have been derived from mice and, more recently, from non-human primates and humans (Evans et al. (1981) *Nature* 292: 154-6; Matsui et al. (1991) *Nature* 353: 750-2; Thomson et al. (1995) *Proc. Natl. Acad. Sci. USA* 92: 7844-8; Thomson et al. (1998) *Science* 282: 1145-7; and Shambloott et al. (1998) *Proc. Natl. Acad. Sci. USA* 95: 13726-31).
- 20 Stem cells have been identified in most organs and tissues. The best-characterized is the hematopoietic stem cell, which can give rise to any of the different types of terminally differentiated blood cells. This is a mesoderm-derived cell, purified based on cell surface markers and functional characteristics (Hill et al. (1996) *Exp. Hematol.* 24: 936-43).
- 25 Also well-characterized are the neural stem cell and a number of mesenchymal stem cells derived from multiple sources (Flax et al. (1998) *Nature Biotechnol.* 16: 1033-1039; Clarke et al. (2000) *Science* 288: 1666; Bruder et al. (1997) *J. Cell. Biochem.* 64: 278-294; Yoo et al. (1998) *J. Bone Joint Surg. Am.* 80: 1745-1757; Makino et al. (1999) *J. Clin. Invest.* 103: 697-705; and Pittenger et al. (1999) *Science*
- 30 284: 143-147).

The term "adult stem cells and progenitor cell populations" is to be understood, in accordance with the present invention, as meaning cells that can be derived from any source of adult tissue or organ, can replicate as undifferentiated cells, and have the potential to differentiate into at least one, preferably multiple, cell lineages.

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"High levels of telomerase activity" can be correlated to the two-fold level observed in the immortal human cell line MCF7 (Soule et al. (1973) J. Cancer Inst. 51: 1409-1416).

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"In vitro" means, for the purposes of the present invention, a conversion, i.e. a reaction, which proceeds outside a live organism.

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"Regenerative tissue" is a mammalian, preferably human, fluid, tissue or organ, which is preferably selected from the group consisting of peripheral blood, cord blood, bone marrow, brain, skin, retina, hair papilla, muscle, liver, pancreas and intestine. As already mentioned, human intestinal epithelia derived from the small intestine are most preferred. Such intestinal stem cells reside just above the crypt base in the small intestine and/or at the crypt base in the colon.

20

These observations, as also indicated in the examples, demonstrate that adult human stem cells, preferably derived from intestinal epithelia, give rise to cells that can form derivatives of all three germ layers. In addition, these cells share some of the properties of embryonic stem cells. Thus, HISC can overcome their ectodermal commitment and show a very broad plasticity that is not restricted to ectodermal derivatives.

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The preferred HISC, in spite of being adult stem cells, have an enormous capacity to proliferate. These cells preferably can divide at least 50-80 times, more preferably 60-70 times, without losing their ability to proliferate and differentiate. After about 15 passages, the cells may become senescent and telomerase activity may decrease. Changes in the environment could have an influence on loss of potency; it has been

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calculated that during a human life-time, approximately 5,000 intestinal stem-cell divisions occur (Booth and Potten (2000) J. Clin. Invest. 105: 1493).

Furthermore, the invention relates to a pluripotent adult stem cell or pluripotent adult stem cell population, wherein the cell can be induced to differentiate to form at least one differentiated cell type of mesodermal, ectodermal and endodermal origin.

- 5 Therefore, the pluripotent adult stem cell or stem cell population can be used to generate differentiated cell lineages. The differentiated cell types can be identified or selected by any method known in the art. For instance, lineage specific markers can be used for sorting methods such as fluorescent activated cell sorting (FACS) or other surface antigen based enrichment. Other examples include DNA chip technology, which  
10 can be used to detect lineage-specific genes, or reverse transcription polymerase chain reaction (RT-PCR), which can be used to analyze lineage-specific gene expression.

The object is preferably achieved by a method for selection and differentiation of a pluripotent adult stem cell population to a desired differentiated cell population,  
15 comprising the steps of: a) isolating a population derived from regenerative tissue containing somatic adult stem cells; b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer; c) selecting and replating colonies which have a pluripotent morphology; d) causing differentiation by deleting the fibroblast feeder layer; and e) selecting the cells of said desired cell lineage.

- 20 In a preferred embodiment, such a differentiated cell lineage can be obtained by selecting and differentiating a pluripotent adult stem cell population to a desired differentiated cell population, by: a) isolating a population derived from regenerative tissue containing somatic adult stem cells, preferably human; b) plating and culturing the  
25 cells of a) on mitotically inactivated fibroblast feeder layer, preferably human; c) selecting and replating colonies which have a pluripotent morphology; d) transfecting cells in selected colonies to introduce a marker gene that enables the selection of one desired cell lineage from the other cell lineages that result from differentiation of the stem cells; e) causing differentiation by deleting the fibroblast feeder layer and culturing  
30 at high density in suspension; and f) selecting the cells of said desired cell lineage

based on the marker gene. The desired cell lineage for example can be neuronal, myocardial, hepatic, pancreatic, pancreatic islet, renal, or dermal.

The marker gene is under the control of a promoter that causes expression of the marker gene to enable selection of one desired cell lineage from other cell lineages. This is achieved by using a promoter which is activated only in the desired cell lineage. Furthermore, the marker gene can confer antibiotic resistance, wherein selection includes contacting the mixed differentiated cellular population with an antibiotic to which cells of the desired cell lineage have been conferred resistance by the expression of the first marker gene, thereby killing cells of other cell lineages, but not cells of the desired one cell lineage that express the marker gene.

The promoters listed in Table 1 are useful for the selection of specific cell lineages:

Table 1. Cell Lineage-Specific Promoters

Promoter	Obtained Cell Lineage	Literature
$\alpha$ -cardiac myosin heavy chain promoter	cardiomyocytes	Klug et al. (1996) J. Clin. Invest. 98:216-24
human atrial natriuretic factor (hANF) promoter	cardiomyocytes	Wu et al. (1989) J. Biol. Chem. 264:6472-79
SOX2 gene	neural precursors	Li et al. (1998) Curr. Biol. 8:971-4
Human insulin promoter (HIP)	insulin-secreting cells	Soria et al. (2000) Diabetes 49:157
p63	keratinocyte stem cells	Pellegrini et al. (2001) Proc. Natl. Acad. Sci. USA 98:3156-3161; Li et al. (1998) Proc. Natl. Acad. Sci. USA 95:3902-3907; Zhu et al. (1999) Devel. 126:2285-2298
C8/144B	hair follicle stem cells	Lyle et al. (1998) J. Cell Science 111:3179-3188
Msi-1	neuronal cells	Sakakibara et al. (1997) J. Neurosci. 17:8300-8312

The cell preparation and process for the differentiation and selection thereof is disclosed in WO 95/14079, which is encompassed in the disclosure of the present invention.

Therefore a further aspect of the invention relates to a method for the selection and differentiation of pluripotent adult stem cells to a desired differentiated cell lineage comprising the steps a) – f), by using the indicated promoter (as in Table 1) for obtaining the desired differentiated cell lineage.

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In another preferred embodiment, a differentiated cell lineage can be obtained by selecting and differentiating a pluripotent adult stem cell population to a desired differentiated cell population by performing steps a) - c) as above, and then, as step (d), transfecting cells in selected colonies to introduce a lineage-specific gene for the purpose of driving differentiation toward a chosen cell lineage, and a marker gene for the purpose of selection. The cells or cell population can then be differentiated and selected according to steps e) and f).

10

The marker gene can confer antibiotic resistance, wherein selection includes contacting the mixed differentiated cellular population with an antibiotic to which cells of the desired cell lineage have been conferred resistance by the expression of said lineage-specific gene, thereby killing cells of the other cell lineages, but not cells of the desired, one cell lineage that express the lineage-specific gene.

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For instance, the following constructs can be used to drive undifferentiated stem cells toward a pancreatic, insulin-secreting fate: CMV-pdx, L32-pdx,  $\beta$ -actin-pdx, CMV-cdx, hIns-pGK-hygro, where CMV (cytomegalovirus), L32 and  $\beta$ -actin are constitutive promoters, and pdx (pancreas/duodenum homeobox) and hIns (human insulin) are pancreatic cell-specific genes. Suitable genes for these constructs are described for instance in the literature and are known to those of skill in the art. See e.g., Sakakibara et al. (1996) Dev. Biol. 176:230-242; and Watt (1998) Phil. Trans. R. Soc. Lond. B 353:831-837; James et al. (1991) J. Biol. Chem. 266:3246-3251; Lorentz et al. (1997) J. Cell Biol. 139:1553-1565; Suh et al. (1996) Mol. Cell. Biol. 16:619-625; Silberg et al. (1997) Gastroenterol. 113:478-486; and Yamada et al. (2001) Am. J. Physiol. Gastrointest. Liver Physiol. 281:G229-G236. The differentiated and selected  $\beta$  cells can be tested for quality and characterized using the parameters listed in Table 2.

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Table 2. Parameters for Pancreatic  $\beta$  Cell Quality Control

Insulin secretion	Insulin processing	Islet hormone profile	Exocrine pancreas profile	Transcription factor profile	Metabolic profile	Enzyme profile	Electrophysio. profile	Cell prolif. profile	Apoptosis profile
Static or dynamic	Pre-insulin/insulin ratio	Glucagon	Amylase	PDX-1	Glucose usage/oxidation	Glut2	SUR-1	GM	Chromatin condensation
Modulation by: glucose nutrition hormones neurotransmitters	Content & secretion	Somatostatin	Lipase	Isl-1		GK	Kir6.1	GLP-1	Bcl-2 in response to IL-1 $\beta$
	Total insulin content	PP		HNF-4		GPDH	Ca <sup>2+</sup>	Insulin	IFN $\gamma$
		GABA		HNF-1 $\alpha$ $\beta$ 2		LDM	pH	IGF I	TNF-1
				Pax6		FAS	pharmacological modulation	IGF II	
				Nkx2.2		HK-II			
				Nkx6.1		GAD65			
				IB1		Catalase			
				c-myc		SOD			
						GSM-peroxidase			

Pluripotent adult stem cells can also be differentiated by any method known in the art. For instance, co-culture with differentiated cells, gene knockout, and the addition of growth factors or other modification of culture conditions can be used in the differentiation of stem cells.

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Another subject matter of the invention is a drug product which comprises pluripotent adult stem cells as mentioned before and/or differentiated cells as mentioned before wherein the differentiated cells are selected from the group consisting of neuronal, myocardial, hepatic, pancreatic (including islet cells), renal and/or dermal cells. The drug product preferably further comprises suitable additives, adjuvants and/or a pharmaceutically acceptable vehicle. The drug product can be used for treatment of diseases such as diabetes, neuronal diseases, liver diseases, heart diseases and/or autoimmune disorders. Subject matter of the invention is further a process for the preparation of such a drug product.

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Another subject matter of the present invention is a method of treating a disease, wherein pluripotent cells as mentioned before or differentiated cells as mentioned before or a composition containing these cells are administered to a patient in need thereof. The differentiated cell or cell population is here preferably a neuronal, myocardial, hepatic, pancreatic (including islet cells), renal, or dermal differentiated cell or cell population. The disease can be diabetes, neuronal disease, liver disease, heart disease or autoimmune disorders. The therapy can be human cell or gene therapy, preferably in transplantation therapy.

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The invention additionally relates to providing a diagnostic composition comprising the pluripotent cell or cell population or the differentiated cell or cell population or a composition comprising at least one of these cells or cell populations. The composition may contain a pharmaceutically acceptable vehicle. By "vehicle" is meant a carrier for the administration of differentiated cells, preferably sterile isotonic saline. The composition may also contain reagents for determining the physiological or morphological response

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of the differentiated cells or cell line to mutagenic, toxic or beneficial agents or treatments.

Description of the drawings

5 Figure 1 shows adult human stem cells derived from gut epithelium. Scale bars = 100  $\mu\text{m}$ . (A) Phase contrast image of human intestinal epithelial cells isolated from fresh small intestine specimens obtained from cadaveric donors. (B) Phase contrast image of an HISC-2 colony showing pluripotent-like morphology after 23 days of culture. (C) HISC-2 colony (indicated by arrow) showing histochemical staining for alkaline  
10 phosphatase. The fibroblast feeder layer (indicated by triangle) was always negative for alkaline phosphatase staining. (D) Phase contrast image of an EB formed 6 days after culturing HISC-2 cells at high density, in plastic Petri dishes, in the absence of fibroblasts and rhLIF. (E) Some adult human stem cells stained with an antibody recognizing the SSEA-3 epitope.

15 Figure 2 shows some adult human stem cells stained with an antibody recognizing the SSEA-4 epitope (A) and a phase contrast view of the same field of cells (B).

Figure 3 shows alkaline phosphatase staining of a human intestinal stem cell EB (A)  
20 and a negative control (B).

Figure 4 shows that adult human stem cells derived from gut epithelium differentiate into different types of cells. Scale bars = 100  $\mu\text{m}$ . Adult human stem cells after 6 days formed EBs and were subsequently differentiated by plating at high density, in the  
25 absence of fibroblasts, on gelatin-treated tissue culture plates. After 1-3 weeks of culture in these conditions a variety of differentiated cells appeared. Among the structures identified were (A) hyaline-like structures (indicated by arrow), (B) neural ganglionic-like structures with elongated processes that extended out from their cell bodies forming networks (indicated by arrow), and (C) secretory glandular-like  
30 structures.



Figure 5 shows marker expression in the differentiated progeny of adult human stem cells. Scale bars = 50  $\mu$ m. Differentiated HISC-2 cells strongly reacted in immunocytochemistry assays with monoclonal antibodies (mAb) against ectoderm proteins, such as neurofilament-200 (A), syntaxin (B), and glial fibrillary acidic protein (C), and against mesoderm proteins, such as vimentin (C), actin (D), and desmin (E). Lower immunoreactivity was observed for endoderm products, such as  $\alpha$ -fetoprotein (F). Cells showing immunoreactivity for both ectoderm and mesoderm are present in (C).

- 10 Figure 6 shows G418 selection of human intestinal stem cells using a human atrial natriuretic factor (hANF) promoter (A) and a human insulin (hIns) promoter (B).

The following examples and figures will assist those skilled in the art to better understand the invention and its principles and advantages. The following examples and figures are intended to illustrate the invention, not to limit the scope thereof.

#### Example 1 HISC Isolation and Culture

##### 20 **Isolation of adult human intestinal epithelium**

Five specimens of adult (between 25 and 50 years old) human small intestine (a 2-3 cm piece of duodenum) were obtained from cadaveric donors (Table 3). First, specimens were thoroughly rinsed in 100 ml of sterilized phosphate buffered saline (PBS) (Gibco-BRL) at 37°C with a syringe. The mucosa was dissected from the submucosa, cut into strips that were rinsed again in 100 ml of sterilized PBS, supplemented with 250 ng/ml amphotericin B (Sigma), at 37°C, and then incubated in 20 ml of PBS, supplemented with 250 ng/ml amphotericin B and containing 10 mM dithiothreitol (Sigma), for 30 minutes at room temperature. Afterwards, the strips were rinsed again in 100 ml of sterilized PBS supplemented with 250 ng/ml amphotericin B, at 37°C and then incubated for 45 minutes in 1 mM EDTA (Sigma) plus 0.25% trypsin (Gibco-BRL) in Dulbecco's modified essential medium (DMEM) (Gibco-BRL) at 37°C. Finally, epithelial cells were detached by gentle shaking of the vessel. The cells suspended in the

medium were immediately filtered through an 80- $\mu$ m nylon mesh and the mesh was rinsed with 20 ml of DMEM.

### **Isolation and culture of pluripotent cells from human intestinal epithelia.**

#### **5    Fibroblast Feeder Cells**

A line of human fibroblasts was isolated from the piece of duodenum of a donor. For isolating this cell line,  $10^7$  cells obtained from the previous protocol, were plated onto 100-mm tissue culture dishes (25020-100, Corning) and cultured in DMEM supplemented with 10% fetal bovine serum (Gibco-BRL), 2 mM glutamine (Gibco-BRL),  
10    100 IU/ml penicillin (Gibco-BRL), 0.1 mg/ml streptomycin (Gibco-BRL) and 250 ng/ml of amphotericin B (Sigma). Every 48 hours, the culture medium was changed. After ten days of culture, fibroblast growth was evident. Then, human fibroblasts were selected, transferred to new dishes and expanded by culturing in the fibroblast culture medium. For each passage,  $5 \times 10^6$  fibroblasts were plated onto 100-mm tissue culture dishes,  
15    cultured in the same fibroblast culture medium, and grown to confluency (3-5 days). The medium was changed every 24 hours. When confluent, the fibroblasts were trypsinized and split.

Finally, to inactivate confluent human fibroblasts, they were incubated for 3 hours at  
20    37°C in the fibroblast culture medium in the presence of 10  $\mu$ g/ml of mitomycin C (Sigma).

#### **Intestinal Stem Cells**

$5 \times 10^6$  isolated epithelial cells (see previous protocol) were plated onto 60-mm tissue  
25    culture dishes (25060-60, Corning) and cultured on inactivated human fibroblast feeder layer ( $75.000 \text{ cells/cm}^2$ ), in DMEM supplemented with 15% fetal bovine serum, 0.1 mM nonessential amino acids (Gibco-BRL), 0.1 mM 2-mercaptoethanol (Gibco-BRL), 2 mM glutamine, 500 ng/ml human recombinant fibroblast growth factor-2 (rhFGF-2) (Upstate Biotech), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 250 ng/ml amphotericin B and  
30    1,000 units/ml of human recombinant leukemia inhibitory factor (rhLIF) (Sigma). After 8 hours of culture, the culture medium was changed. Then, during the subsequent week,

the culture medium was changed every 8 hours. Afterward, the culture medium was changed every 24 hours.

Initially, isolated intestinal epithelial cells died rapidly, but after 3 days of culture, 27% of the cells remained as monolayers. To assess the purity of these cells, they were stained for the epithelial cell marker, cytokeratin-18, with specific primary mAb (Sigma) and localized with fluorescent (TRITC) secondary antibody (Sigma) by confocal microscopy. The percentage of cells positive for cytokeratin-18 was determined by immunocytochemistry to be 95%.

During the following three weeks, epithelial cells continued proliferating. After this time, small colonies with a morphology resembling pluripotent stem cells were occasionally observed growing from the intestinal epithelial cell cultures. These cells were selected, transferred to new dishes and expanded by culturing in the same culture medium. For each passage,  $2 \times 10^6$  cells were cultured in tissue culture flasks (Falcon 75 cm<sup>2</sup>, 353084, Becton Dickinson), on new inactivated human fibroblast feeder layer (75.000 cells/cm<sup>2</sup>), in the same culture medium, and grown to confluence (5-7 days). The medium was changed every 48 hours. When confluent, the cells were trypsinized and split. Using this methodology, four clones were established (clones: HISC 1 to 4; Table 3). Although the four clones showed similar characteristics, only data from clone HISC-2 are shown.

Table 3. Profile of Intestinal Tissue Donors

Patient	Age	Sex	Death cause	Time in ICU (h)	Time from extraction to isolation (h)
First (HISC-1)	40	Male	Stroke	96	4
Second (no line)	50	Female	Stroke	96	4.30
Third (HISC-2)	32	Male	Accident	15	2.20
Fourth (HISC-3)	39	Male	Accident	10	2.45
Fifth (HISC-4)	20	Male	Accident	18	3.50

Example 2  
HISC Characterization

Throughout the culture period in the undifferentiated stage (passages 2-12), HISC-2 cells showed some pluripotent stem cell-like characteristics such as a pluripotent cell-like morphology (Figure 1B), high alkaline phosphatase activity staining (Figure 1C), a high nuclear to cytoplasm ratio, a high proliferation rate (doubling time = 15 h), the expression of SSEA-3 (Figure 1E), SSEA-4 (Figure 2A) and Oct-4. Alkaline phosphatase was detected with Vector Blue substrate (Vector Labs) and SSEA-3 was detected by immunocytochemistry with specific primary mAb (Hybridoma Bank) and localized with fluorescent (TRITC) secondary antibody (Sigma) by confocal microscopy. Immunological screening for the presence of other known stem cell markers, such as SSEA-1, SSEA-2, GCTM-2, TRA-1-60 and TRA-1-81, can also be used to characterize pluripotent stem cells. Shamblo et al. (1998) Proc. Natl. Acad. Sci. USA 95:13726-31.

Early passages (2-12) of HISC-2 cells also had a high telomerase activity (twice that observed in the immortal human cell line MCF7). Telomerase activity was measured with the TRAP assay. Cells were lysed as previously described. For TRAP analysis, the reaction mixture was performed in 0.2 ml thin-walled tubes, in a 10 µl volume containing 50 µM dNTPs, TRAP buffer (final concentrations of 20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 68 mM KCl, 0.05% Tween<sup>®</sup>, 20.1 mM EGTA), 100 ng TS primer (AATCCGTCGAGCAGAGTT) (SEQ ID NO: 1), 100 ng CX primer (CCCTTACCCTTACCCTTACCCTAA) (SEQ ID NO: 2) labeled with HEX phosphoramidites (Perkin-Elmer Applied Biosystems), 2.5 U AmpliTaq Gold (Perkin-Elmer) and 1 µg of protein cell lysate. The tubes were incubated at room temperature for 20 min, then heated to 95°C for 10 min, followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec, with a final extension step at 72°C for 1 min in a Techne Thermal Cycler. For gel analysis, an electrophoresis was carried out in an ABI PRISM 377 DNA sequencer (Perkin-Elmer). The samples were run in the GS 36C-2400 module for 3 h. DNA fragment analysis was performed using GeneScan 2.1 software (Applied Biosystems).

Other methods for characterization of isolated HISC can also be employed. Telomere length can be determined by Southern blotting for telomere restriction fragment. Quantitative RT-PCR can be used to confirm the origin and character of isolated stem cells by detecting expression of embryonic transcription factors such as TCF4, Sox2 and Oct-4. In addition, teratoma studies can be performed to demonstrate the pluripotency of cells. This data can be used to distinguish HISC from intestinal epithelial non-stem cells

### Example 3

#### HISC Differentiation into Multiple Cell Types

When allowed to differentiate in vitro by culturing at high density, in plastic Petri dishes, in the absence of fibroblasts and rhLIF and in the same medium as that used for initial culture, HISC-2 cells (passages 2-12) formed EBs (Figure 1D). Subsequent in vitro differentiation, by plating trypsinized EBs at high density, in the absence of fibroblasts, on gelatin-treated tissue culture plates, in the last mentioned medium, for three to six days, revealed a variety of differentiated cells. Thus, in vitro differentiation occurred when HISC-2 cells were cultured in the absence of embryonic fibroblasts and at a high density, both in the presence and absence of rhLIF.

After 1-3 weeks of culture in these conditions, a variety of differentiated cells appeared. Hyaline structures (Figure 4A), neural ganglionic-like structures with elongated processes that extended out from cell bodies forming networks (Figure 4B), secretory glandular structures (Figure 4C) and contracting muscle, pacing at frequencies of 30-70 pulses/min., were observed.

Immunophenotyping of these cells was also carried out. Differentiated HISC-2 cells reacted with antigens characteristic of all three embryonic germ layers in immunocytochemistry experiments using specific primary mAbs (Sigma), localized with fluorescent (TRITC or FITC) secondary antibodies (Sigma) and visualized by confocal microscopy. So it could be shown that differentiated HISC-2 cells strongly expressed the ectoderm proteins neurofilament-200 (Figure 5A), syntaxin (Figure 5B) and glial fibrillary acidic protein (Figure 5C) and the mesoderm proteins vimentin (Figure 5C),

actin (Figure 5D) and desmin (Figure 5E), and displayed lower immunoreactivity for the endoderm product  $\alpha$ -fetoprotein (Figure 5F). As observed in Figure 5C, some cells expressed markers from two germ layers (mesoderm and ectoderm).

- 5 When culturing HISC-2 cells after passage 15, the cells lost telomerase activity and showed a changed morphology and decreased proliferation rate (doubling time = 44 h). The total expansion capacity of HISC-2 cells was approximately  $4 \times 10^{11}$ .

#### Example 4 Differentiated Cell Selection

10 Described is a genetic enrichment approach that facilitates the generation of highly enriched cultures of cardiomyocytes from differentiating intestinal derived pluripotent cells. The approach relies on the use of two transcriptional units, introduced on a common vector backbone into undifferentiated intestinally derived pluripotent cells.

15 The first transcriptional unit comprises a promoter expressed in undifferentiated stem cells, linked to a marker gene suitable for enrichment of cells carrying the DNA. In this example, the phosphoglycerate kinase promoter and a cDNA encoding resistance to hygromycin were used; the transcriptional unit is designated pGK-hygror. The second  
20 transcriptional unit comprises a cell lineage-restricted promoter, linked to a marker gene suitable for enrichment of the desired cells. In this example, the cardiomyocyte-restricted  $\alpha$ -cardiac myosin heavy chain promoter and a cDNA encoding aminoglycoside phosphotransferase were used; the transcriptional unit is designated MHC-neor.

25 Undifferentiated, intestinally derived pluripotent cells are transfected with the MHC-neor/pGK-hygror construct. Cells incorporating the DNA are enriched based on their resistance to hygromycin. Differentiation is then induced, and once evidence of cardiomyogenesis is observed (i.e. spontaneous contractile activity), the cultures are  
30 treated with G418. Since the  $\alpha$ -MHC promoter is only active in cardiomyocytes, only these cells express aminoglycoside phosphotransferase and survive G418 treatment.

The genetic enrichment approach has the advantage that very long-term cultures of terminally differentiated cardiomyocytes can be generated, since all non-cardiomyocytes are eliminated from the culture. Moreover, the approach is easily amenable to gene transfer, either prior to differentiation or after the generation of terminally differentiated cells. In addition, the genetic enrichment approach is applicable to all pluripotent stem cell systems.

The Methods are divided into sub-sections describing: (1) the transfection and selection of undifferentiated intestinally derived pluripotent cells; (2) the "en mass" differentiation of the selected cells; and (3) the generation of highly enriched cardiomyocyte cultures.

### 1. Transfection and selection of undifferentiated HISC:

1.1. Prior to transfecting intestinally derived pluripotent cells, the selection cassette (pMHC-neor/PGK-hygror) was digested with Xho I/Hind III and the fragment containing the entire MHC-neor/PGK-hygror sequence was isolated using a gene clean kit.

1.2. Intestinally derived pluripotent cells are maintained in an undifferentiated state by culturing on mitotically inactivated human fibroblast feeder layer in Growth Medium: DMEM (Gibco-BRL) supplemented with 15% fetal bovine serum (Gibco-BRL), 0.1 mM nonessential amino acids (Gibco-BRL), 0.1 mM 2-mercaptoethanol (Gibco-BRL), 2 mM glutamine (Gibco-BRL), 500 ng/ml human recombinant fibroblast growth factor-2 (rhFGF-2) (Upstate Biotech) and 1,000 units/ml of human recombinant leukemia inhibitory factor (rhLIF) (Sigma).

1.3. Cells were dissociated using trypsin, counted, and  $4 \times 10^6$  cells were resuspended in 0.8 ml of Growth Medium. Cells were then transferred into an electroporation chamber and kept on ice.

1.4. MHC-neor/PGK-hygror DNA (1  $\mu$ g) were mixed with salmon testes DNA (25  $\mu$ g) in a total volume of 70  $\mu$ l and sonicated. This mixture was added to the cells and left in the electroporation chamber on ice for 15 minutes.

1.5. Cells were electroporated (180 volts, 800  $\mu$ F) and left on ice for 15 minutes.

1.6. Cells were plated in 100 mm Corning dishes ( $6 \times 10^5$  cells/dish) in Growth Medium for 24 hours.

1.7. The next day, the medium was removed by aspiration and the cells were switched to Growth Medium supplemented with hygromycin B (200 µg/ml).

1.8. Medium was changed daily and transfected cells were selected over a period of 7 days. Cells can be trypsinized and replated into new dishes if a plate becomes

5 confluent.

## 2. "En mass" differentiation of transfected HISC

2.1. After 7 days of hygromycin selection, cells were dissociated using trypsin and 4 X 10<sup>6</sup> cells were plated in a 100 mm bacterial Petri dish in 10 ml of Differentiation Medium: 10 DMEM (Gibco-BRL) supplemented with 15% fetal bovine serum (Gibco-BRL), 0.1 mM nonessential amino acids (Gibco-BRL), 0.1 mM 2-mercaptoethanol (Gibco-BRL), 2 mM glutamine (Gibco-BRL), 500 ng/ml human recombinant fibroblast growth factor-2 (rhFGF-2) (Upstate Biotech). Cells grow in suspension under these conditions.

2.2. The cells were supplemented with 5 ml of Differentiation Medium on the next day 15 to facilitate EB formation.

2.3. On the third day, medium containing EBs was transferred, using a 10 ml pipette, into a sterile 50 ml cell culture tube and EBs were allowed to settle by gravity. Medium was then aspirated, and EBs were resuspended in 10 ml of fresh Differentiation Medium and plated in a new Petri dish.

2.4. Cells were then supplemented with 5 ml of Differentiation Medium on the next day. 20

2.5. EBs were collected on the fifth day by gravity and resuspended in 10 ml of Differentiation Medium. EBs were plated in 100 mm Corning cell culture dishes at different dilutions (1:2, 1:5, 1:10 etc).

2.6. The medium was changed daily. Regions of cardiogenesis could be readily 25 identified by the presence of spontaneous contractile activity within 4-6 days of EB attachment.

## 3. Selection of cardiomyocyte restricted lineages:

3.1. For enrichment of cardiomyocyte restricted lineages, cultures exhibiting 30 spontaneous contractile activity were grown in Differentiation Medium plus G418 (200 µg/ml).



3.2. Cultures can be grown in such a Differentiation Medium for as long as required to eliminate non-cardiomyocytes.

#### Example 5

#### Selective Differentiation of HISC

Described is the generation of highly enriched cultures of pancreatic  $\beta$  cells from differentiating intestinally derived pluripotent cells. The genetic enrichment approach of Example 4 is modified such that a second expression cassette is introduced to produce a gene product coding for a transcription /growth factor involved in beta-cell differentiation (for example pdx-1). In this example, the first transcriptional unit comprises hINSp-neo-pGK-hygro where hINSp stands for the human insulin promoter and the second transcriptional unit comprises pGK-pdx-1 where pdx-1 represents the human pdx-1 gene.

Undifferentiated, intestinally derived pluripotent cells are transfected with the hINSp-neo-pGK-hygro / pGK-pdx-1 constructs as described in Example 4. Cells which have incorporated the DNA are enriched based on their resistance to hygromycin, and are directed toward an insulin-secreting cell fate by their expression of the pdx-1 gene.

To induce differentiation to an insulin-secreting cell line, hygromycin-resistant HISC cells, obtained by Method 1 of Example 4, are plated onto a 100 mm bacterial Petri dish and cultured in DMEM lacking supplemental LIF. After 8-10 days in suspension culture, the resulting EBs are plated onto plastic 100 mm cell culture dishes and allowed to attach for 5-8 days. For HISC insulin selection, the differentiated cultures are grown in the same medium in the presence of 200  $\mu$ g/ml G418 (GIBCO/BRL). Single G418 EB clones are selected, isolated into 24 well plates, and then expanded. This approach avoids the dilution of robust insulin-secreting clones by clones that are weaker insulin producers. For final differentiation and maturation, the resulting clones are trypsinized and plated in a 100 mm bacterial Petri dish and grown for 14 days in DMEM supplemented with 200  $\mu$ g/ml G418 and 10 mM nicotinamide (Sigma, Madrid). Finally, the resulting clusters (pseudo-islet structures) are cultured for 1-5 days in RPMI 1640

supplemented with 10% fetal bovine serum, 10 mM nicotinamide, 200 µg/ml G418, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 5.6 mM glucose.

Example 6  
Insulin Secretion Assays

Pancreatic  $\beta$  cells produced by the methods of Example 5 can be screened for insulin secretion. For static incubations, cells are plated in 12-well dishes (Corning Glass Works, Corning, NY) at a density of 250,000 cells per well and allowed to grow overnight in the same RPMI1640 indicated above. Cells are washed twice, for 10 min each, in fresh modified Krebs buffer with 0.1% bovine serum albumin (BSA) and 3 mM glucose. The Krebs buffer is kept at 37°C and is constantly gassed with a mixture of O<sub>2</sub> (95%) and CO<sub>2</sub> (5%) to maintain a pH of 7.4. Cells are then transferred to glass vials in free-floating clusters at  $\sim 5 \times 10^4$  cells per vial and incubated at 37°C in 1 ml of the same fresh modified Krebs buffer with 0.5% BSA and with different secretagogues for 30 min. At the end of the incubation period, the buffer is removed from the vials. Each condition is assayed in triplicate. Perfusion studies are performed as described in Perrusa et al. (1999) J. Physiol. 520:470-80. Briefly,  $1 \times 10^5$  HISC-derived insulin-secreting cells (as free floating clusters) are packed into a 1 cm diameter column and sandwiched between two layers of swollen Sephadex G-200 microcarrier beads (Sigma, St. Louis, MO). The column is perfused at a flow rate of 1 ml/min at 37°C with fresh modified Krebs buffer with 1% BSA plus glucose (3 or 22 mM). The HISC-derived insulin-secreting cells are first perfused in 3 mM glucose for 30 min to reach a state of stable insulin release. The solutions are prewarmed at 37°C and continuously gassed. Insulin is assayed by radioimmunoassay (RIA) using the Coat-a-Count kit (DPC, Los Angeles, CA) that detects both rat and human insulin. For measurement of total insulin-cell content, cell pellets are sonicated in 1 mM acetic acid containing 1% BSA and cellular extract is also determined by RIA. Secretion is normalized for cell number by measuring total protein in each experiment by the method of Bradford. Bradford (1976) Anal. Biochem. 72:248-54.

Having thus described in detail the preferred embodiments of the present invention, the invention defined by the appended claims is not to be limited by particular details set

" forth in the above description, as many apparent variations thereof are possible without departing from the spirit or scope thereof.

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## Claims

1. A composition comprising an isolated pluripotent adult stem cell population, derived from regenerative tissue, and having alkaline phosphatase activity, high levels of telomerase activity and the ability to form derivatives of all three embryonic germ layers and/or the ability to form an embryoid body.

2. The composition according to claim 1, wherein the regenerative tissue is selected from the group consisting of peripheral blood, cord blood, bone marrow, brain, skin, retina, hair papilla, muscle, liver, pancreas, epithelium.

3. The composition according to claim 2, wherein the epithelium is derived from the intestine.

4. The composition according to claim 1, wherein the pluripotent adult stem cell population is human.

5. The composition of claim 4, wherein the pluripotent adult stem cell population is a human pluripotent adult intestinal stem cell population.

6. The composition of claim 5, wherein the intestinal stem cell population is derived from the crypt.

7. The composition of claim 4, having stage-specific embryonic antigen-3 (SSEA-3) expression.

8. The composition of claim 1, wherein the pluripotent adult stem cell population is mammalian.

9. The composition of claim 1, wherein the pluripotent adult stem cell population can proliferate in vitro.

10. The composition of claim 1, wherein the pluripotent adult stem cell population can propagate in vitro.

11. A pluripotent adult stem cell, having alkaline phosphatase activity, high levels of telomerase activity and the ability to form derivatives of all three embryonic germ layers, wherein the pluripotent adult stem cell is derived from regenerative tissue.

12. The pluripotent adult stem cell according to claim 11, wherein the regenerative tissue is selected from the group consisting of peripheral blood, cord blood, bone marrow, brain, skin, retina, hair papilla, muscle, liver, pancreas, epithelium.

13. The pluripotent adult stem cell according to claim 12, wherein the epithelium is derived from the intestine.

14. The pluripotent adult stem cell according to claim 11, wherein the pluripotent adult stem cell is human.

5 15. The pluripotent adult stem cell of claim 14, wherein the pluripotent adult stem cell is human.

16. The pluripotent adult stem cell of claim 15, wherein the pluripotent adult stem cell is derived from the crypt.

10 17. The pluripotent adult stem cell of claim 14, having stage-specific embryonic antigen-3 (SSEA-3) expression.

18. The pluripotent adult stem cell of claim 11, wherein the pluripotent adult stem cell is mammalian.

19. The pluripotent adult stem cell of claim 11, wherein the pluripotent adult stem cell can proliferate in vitro.

15 20. The pluripotent adult stem cell of claim 11, wherein the pluripotent adult stem cell can propagate in vitro.

21. The composition according to claim 1, wherein the pluripotent adult stem cell population has the capacity to differentiate to form at least one differentiated cell type of mesodermal, ectodermal and endodermal origin.

20 22. A method for isolating and enriching a pluripotent adult stem cell population, comprising the steps of:

a) isolating a population derived from regenerative tissue containing somatic adult stem cells;

25 b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer;

c) selecting and replating colonies which have a pluripotent morphology.

23. The method according to claim 22, wherein the cells of the selected colonies are expanded over 2-100, preferably over 2-15 passages.

30 24. The method according to claim 23, wherein the cells of the selected colonies are expanded over 10-15 passages.

25. The method according to claim 22 wherein the somatic adult stem cells



are human.

26. The method according to claim 22 wherein the fibroblast feeder layer comprises human cells.

5 27. A method for selection and differentiation of a pluripotent adult stem cell population to a desired differentiated cell population, comprising the steps of:

- a) isolating a population derived from regenerative tissue containing somatic adult stem cells;
- b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer;
- 10 c) selecting and replating colonies which have a pluripotent morphology;
- d) causing differentiation by deleting the fibroblast feeder layer; and
- e) selecting the cells of said desired cell lineage.

28. The method according to claim 27 wherein the somatic adult stem cells are human.

15 29. The method according to claim 27 wherein the fibroblast feeder layer comprises human cells.

30. The method according to claim 27 wherein the cell lineage is selected from the group consisting of neuronal, myocardial, hepatic, pancreatic, pancreatic islet, renal, and dermal.

20 31. A composition comprising the differentiated stem cell population obtained by the method of claim 27.

32. A method for selection and differentiation of a pluripotent adult stem cell population to a desired differentiated cell population, comprising the steps of:

- 25 a) isolating a population derived from regenerative tissue containing somatic adult stem cells;
- b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer;
- c) selecting and replating colonies which have a pluripotent morphology;
- 30 d) transfecting cells in selected colonies to introduce a marker gene that enables the selection of one desired cell lineage from the other cell lineages that result from differentiation of the stem cells;

- e) causing differentiation by deleting the fibroblast feeder layer and culturing at high density in suspension; and
- f) selecting the cells of said desired cell lineage based on the marker gene.

5           33. The method according to claim 32 wherein the somatic adult stem cells are human.

          34. The method according to claim 32 wherein the fibroblast feeder layer comprises human cells.

10          35. The method according to claim 32, wherein the cell lineage is selected from the group consisting of neuronal, myocardial, hepatic, pancreatic, pancreatic islet, renal, and dermal.

          36. A composition comprising the differentiated cell population obtained by the method of claim 32.

15          37. A method for selection and differentiation of a pluripotent adult stem cell population to a desired differentiated cell population, comprising the steps of:

- a) isolating a population derived from regenerative tissue containing somatic adult stem cells;
- b) plating and culturing the cells of a) on mitotically inactivated fibroblast feeder layer;
- 20       c) selecting and replating colonies which have a pluripotent morphology;
- d) transfecting cells in selected colonies to introduce a lineage-specific gene for the purpose of driving differentiation toward a chosen cell lineage, and a marker gene for the purpose of selection;
- 25       e) causing differentiation by deleting the fibroblast feeder layer and culturing at high density in suspension; and
- f) selecting the enriched chosen cell lineage based on the marker gene.

          38. The method according to claim 37 wherein the somatic adult stem cells are human.

30          39. The method according to claim 37 wherein the fibroblast feeder layer comprises human cells.

40. The method according to claim 37, wherein the cell lineage is selected from the group consisting of neuronal, myocardial, hepatic, pancreatic, pancreatic islet, renal, and dermal.

5 41. A composition comprising the differentiated cell population obtained by the method of claim 37.

42. The composition of claim 1, further comprising a pharmaceutically acceptable vehicle.

43. The composition of claim 31, further comprising a pharmaceutically acceptable vehicle.

10 44. The composition of claim 36, further comprising a pharmaceutically acceptable vehicle.

45. The composition of claim 41, further comprising a pharmaceutically acceptable vehicle.

15 46. A method of treating a disease, comprising administering the composition of claim 1 to a patient in need thereof.

47. A method of treating a disease, comprising administering the composition of claim 31 to a patient in need thereof.

48. A method of treating a disease, comprising administering the composition of claim 36 to a patient in need thereof.

20 49. A method of treating a disease, comprising administering the composition of claim 41 to a patient in need thereof.

50. The method according to claim 46, wherein the disease comprises diabetes, neuronal disease, liver disease, heart disease or autoimmune disorders.

25 51. The method according to claim 47, wherein the disease comprises diabetes, neuronal disease, liver disease, heart disease or autoimmune disorders.

52. The method according to claim 48, wherein the disease comprises diabetes, neuronal disease, liver disease, heart disease or autoimmune disorders.

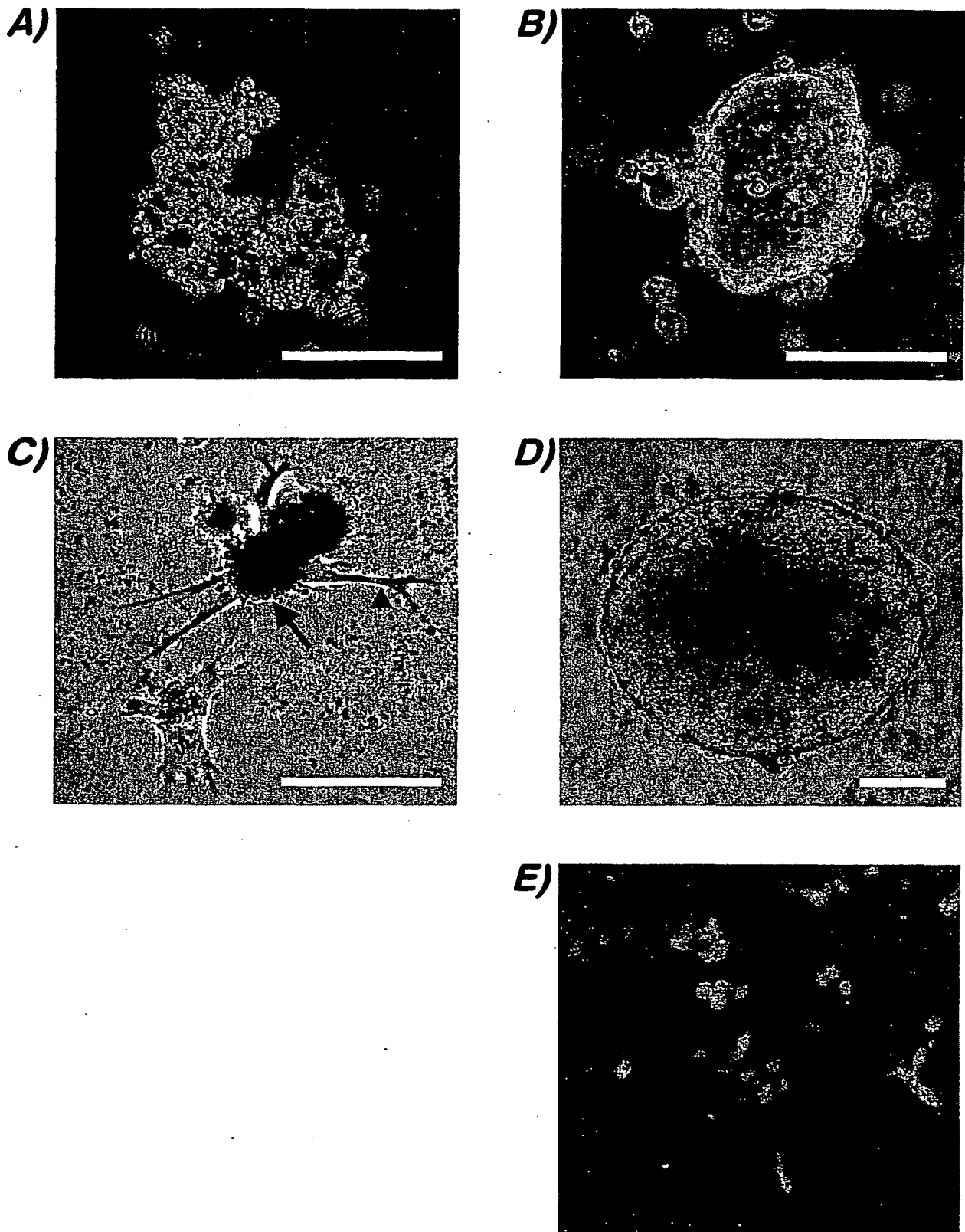
53. The method according to claim 49, wherein the disease comprises diabetes, neuronal disease, liver disease, heart disease or autoimmune disorders.

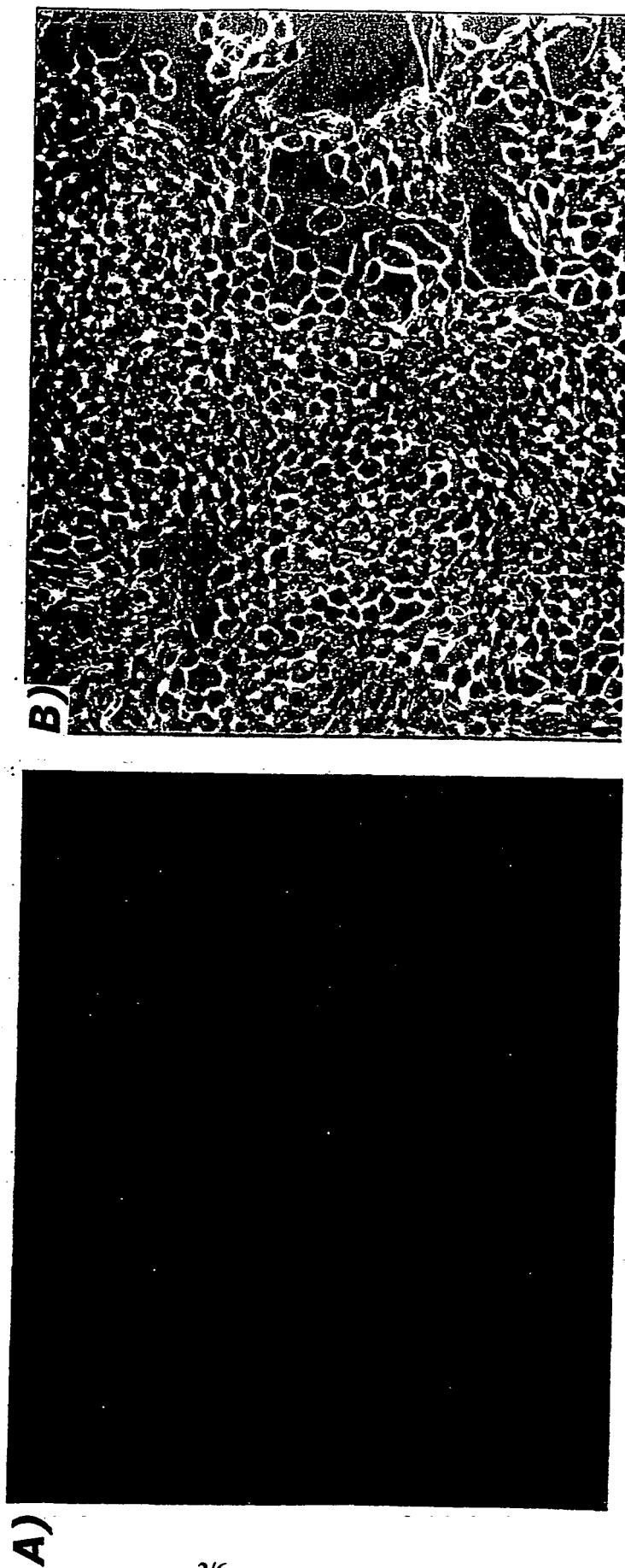
30 54. A diagnostic composition comprising the composition of claim 1.

55. A diagnostic composition comprising the composition of claim 31.

56. A diagnostic composition comprising the composition of claim 36.
57. A diagnostic composition comprising the composition of claim 41.
58. A drug product comprising a composition according to claim 1, 31, 36 and/or 41 and/or a pluripotent adult stem cell according to claim 11.
- 5 59. A process for the preparation of a drug product according to claim 58.

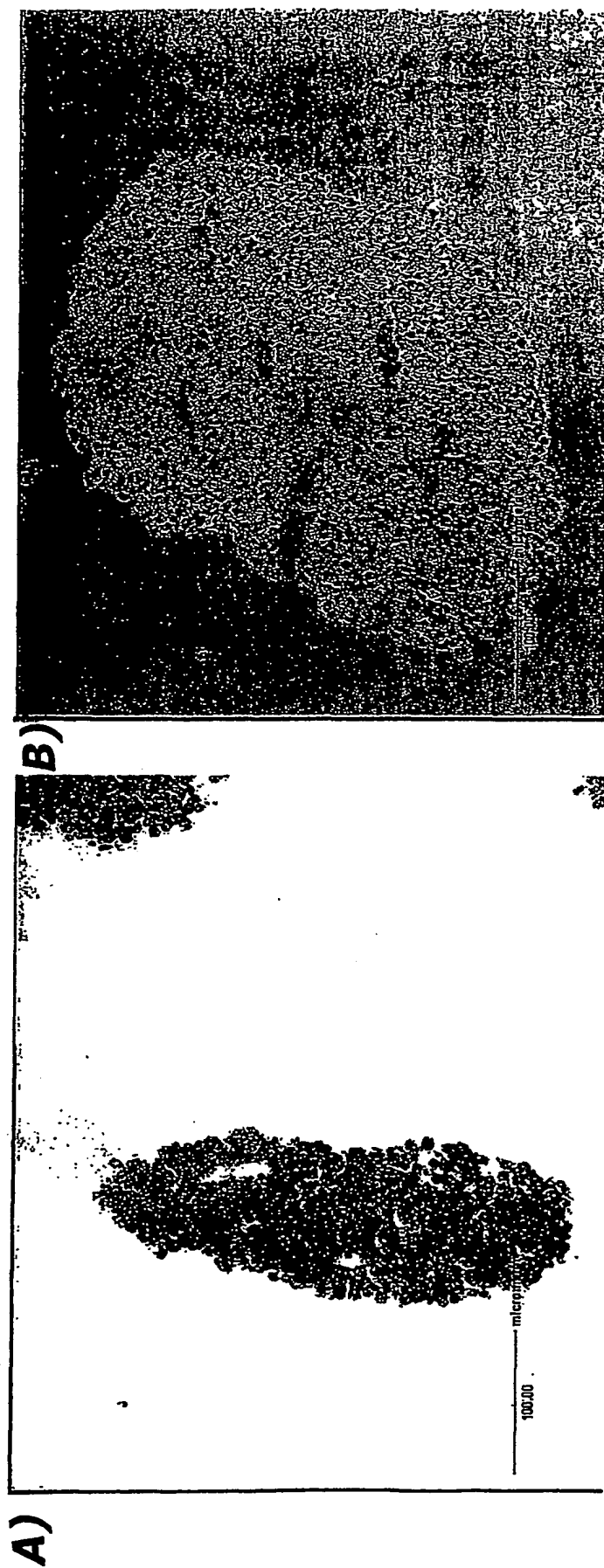
***Fig. 1***





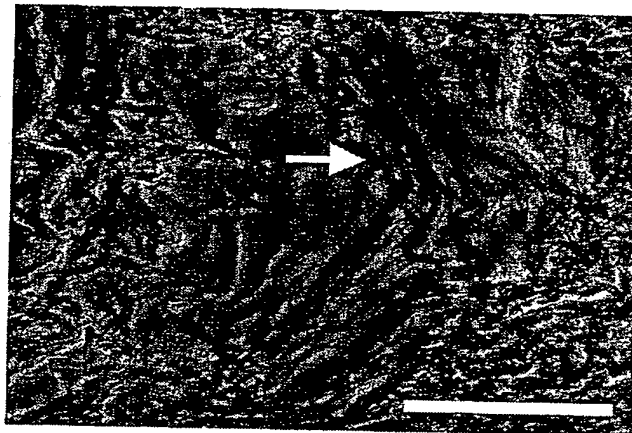
**Figure 2**

**Figure 3**

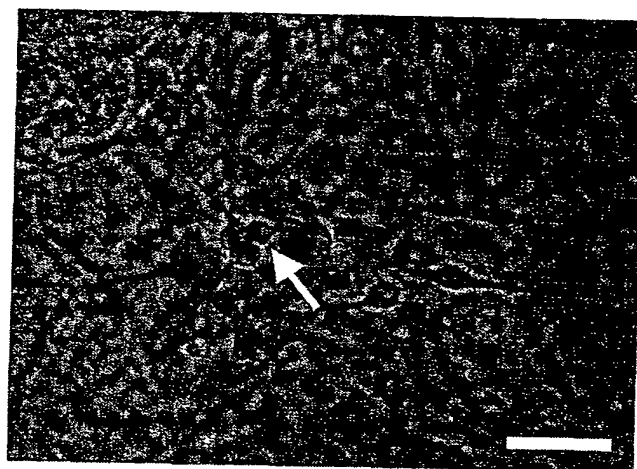


**Fig. 4**

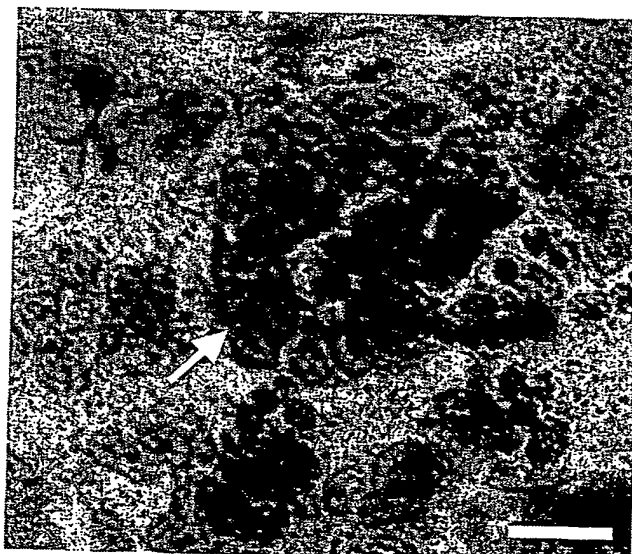
**A)**



**B)**

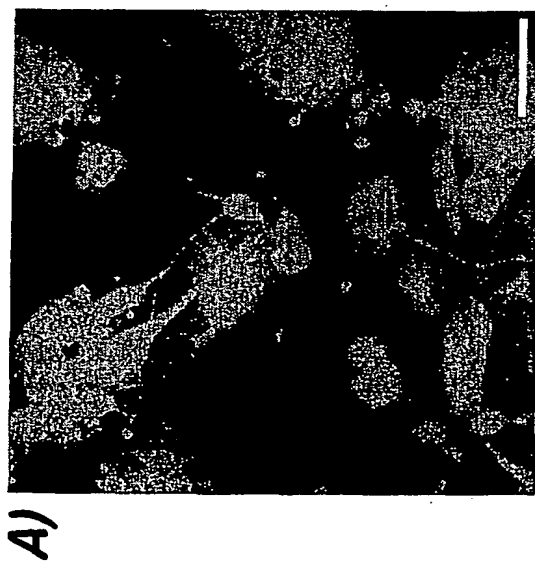


**C)**

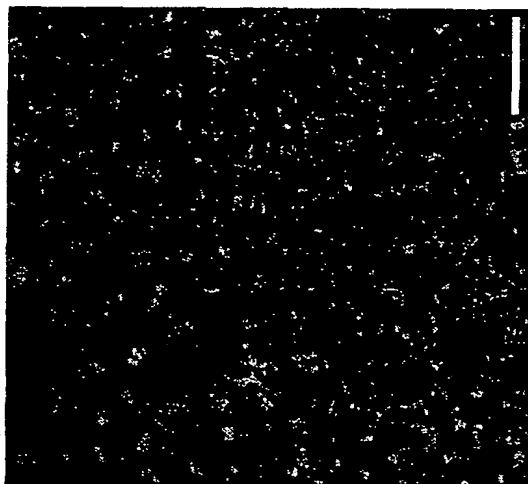




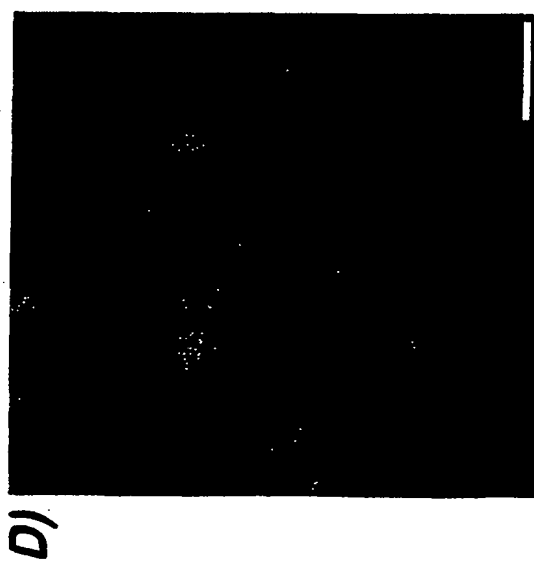
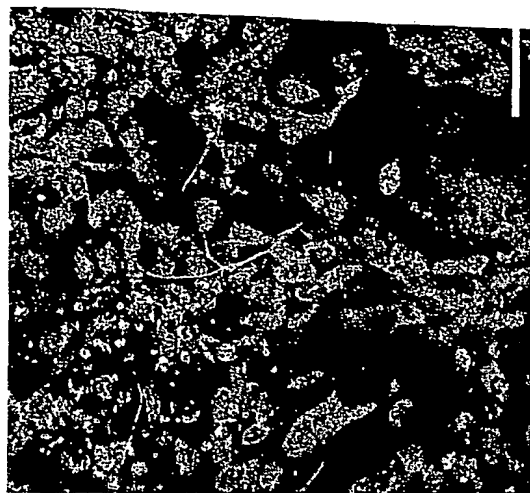
**Fig. 5**



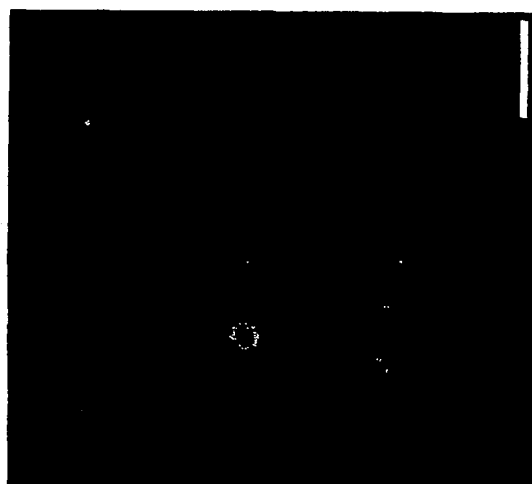
B)



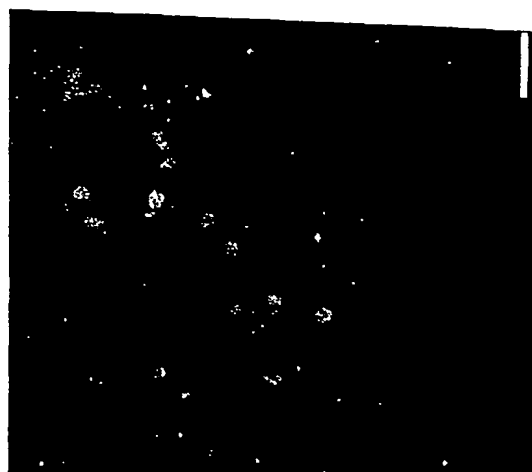
C)



E)

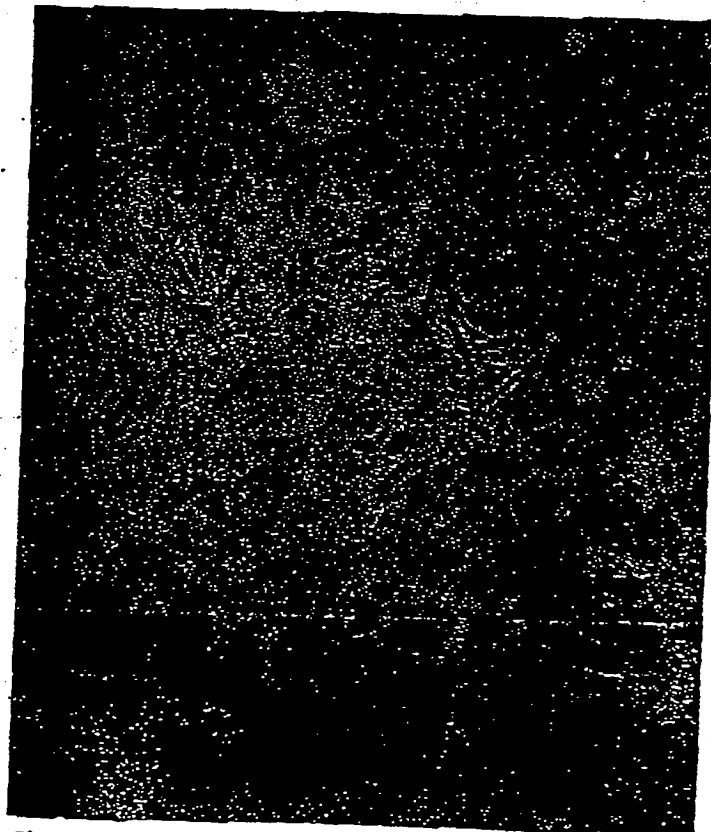


F)



**Figure 6**

**A)**



**B)**

